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Purification of RIP60 and RIP100, Mammalian Proteins with Origin-Specific DNA-Binding and ATP-Dependent DNA Helicase Activities

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Replication of the Chinese hamster dihydrofolate reductase gene (*dhfr*) initiates near a fragment of stably bent DNA that binds multiple cellular factors. Investigation of protein interactions with the *dhfr* bent DNA sequences revealed a novel nuclear protein that also binds to domain B of the yeast origin of replication, the autonomously replicating sequence ARS1. The origin-specific DNA-binding activity was purified 9,000-fold from HeLa cell nuclear extract in five chromatographic steps. Protein-DNA cross-linking experiments showed that a 60-kDa polypeptide, which we call RIP60, contained the origin-specific DNA-binding activity. Oligonucleotide displacement assays showed that highly purified fractions of RIP60 also contained an ATP-dependent DNA helicase activity. Covalent radiolabeling with ATP indicated that the DNA helicase activity resided in a 100-kDa polypeptide, RIP100. The cofractionation of an ATP-dependent DNA helicase with an origin-specific DNA-binding activity suggests that RIP60 and RIP100 may be involved in initiation of chromosomal DNA synthesis in mammalian cells.

The study of DNA replication in a variety of procaryotic chromosomes, bacteriophages, and eucaryotic viruses has permitted the construction of a general model for initiation of DNA synthesis at origins of replication (7). In the general model, initiation is viewed as a stepwise process that involves the ordered interaction of multiple proteins with origin DNA sequences. Origin activation commences with the binding of an initiator protein to origin-specific recognition sequences, which are often tandemly reiterated. Binding of the initiator protein engenders melting at the origin, leading to the formation of a stable presynthesis complex that contains locally unwound DNA (6, 15, 44). The presynthesis complex in turn fosters the assembly of the multienzyme complexes required for bidirectional DNA replication. The timing and frequency of initiation may be regulated by the availability of the initiator protein or by topological perturbations in the template that affect the ability of the initiator protein to unwind origin sequences (5).

Like transcriptional promoters, origins of replication are complex regulatory elements with multiple modular components, including DNA-unwinding elements (51), binding sites for the initiator and accessory factors, and often transcriptional enhancers and promoters (17). In some instances, spacing of origin elements may be critical for function (40, 52); in others, functional components may interact over considerable distances (37, 42). Despite the diversity of organization among origins of replication, unwinding at the origin is likely to be a universal prerequisite for initiation of bidirectional DNA synthesis on duplex DNA templates.

Both DNA sequences and proteins contribute to DNA unwinding at origins of replication. The DNA sequences that facilitate origin unwinding, or DNA-unwinding elements (51), are generally A+T rich and may have special structural properties. Nuclease sensitivity assays show that the A+T-rich regions of the bacteriophage PM2 (30), the yeast 2μm circle (49, 50), and the bacterium *Escherichia coli* (31) origins are readily unwound in response to superhelical stress. Deletion analyses and sequence substitution experiments suggest that DNA sequences that promote helix instability are essential features of the yeast H4 autonomously replicating sequence (ARS) (50) and the *E. coli* origin, *oriC* (31). The role of sequence-directed DNA unwinding in origin activation is likely quite complex, for in yeast cells the propensity for sequences to be readily unwound does not invariably correlate with origin function (27, 29).

Another structural element, stably bent DNA, is a functional component of several replication origins, including those of phage lambda (55), the ARS1 element from yeast cells (45), and the papovavirus simian virus 40 (16). DNA bending, which has been implicated in initiation of DNA synthesis by mutagenesis and sequence substitution studies (16, 53), may promote helix disruption (41), foster the functional interaction of protein-binding sites (56), or serve to uniquely orient the topology of origins for subsequent activation events (33). The spatial relationship of initiator-binding sites to bent DNA has been proposed to represent a highly conserved feature of replication origins (19).

In addition to requiring sequence determinants that promote helix instability, extensive unwinding of origin DNA requires the action of a DNA helicase. The helicase activity may be an inherent property of the initiator protein, as in simian virus 40 large T antigen (48) and the herpes simplex virus UL-9 protein (38), or may consist of a distinct factor that interacts in a specific fashion with the initiator protein-origin DNA complex (4). The helicase involved in initiation may also be required at replication forks during the elongation phase of DNA synthesis and therefore may not represent an initiation-specific replication factor.

Insight into the mechanisms that regulate initiation of DNA synthesis in mammalian cells requires knowledge of the DNA sequences that comprise chromosomal origins of replication and purification of the cognate factors that interact with origin sequences during entry into the S phase. Although extensive genetic, biochemical, and physical evidence suggests that DNA synthesis begins at preferred sites

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in higher eucaryotes (reviewed in reference 51), the lack of a satisfactory functional assay has greatly impeded the isolation of origins of replication from animal cells. Nonetheless, we consider it likely that mammalian origins of replication have structural and functional features in common with their counterparts from other sources and that the mammalian proteins that regulate DNA synthesis encompass the activities attributed to other initiation factors. Therefore, we have examined the origin region of a well-characterized mammalian replicon for DNA sequences and associated proteins that display the properties of other initiation systems.

The replication of the Chinese hamster dihydrofolate reductase gene (*dhfr*) domain has been studied extensively. Pulse-labeling studies in synchronized CHOC 400 cells, which contain approximately 1,000 copies of the *dhfr* replicon (36), show that replication of the amplified *dhfr* genes begins within a doublet of 6.1-kb *Eco*RI fragments termed ELF-F/ELF-F′ (for early-labeled fragments F and F′) (24, 26). The ELF-F/ELF-F′ doublet has been cloned and mapped to region 3′ to the *dhfr* gene (25). Hybridization of replication intermediates formed during the onset of the S phase to cloned ELFs shows that replication of the *dhfr* gene begins within a 4.3-kb *Xba*I fragment that overlaps the ELF-F/ELF-F′ doublet (9). In-gel renaturation analysis of replication intermediates locates initiation events to a 1.8-kb *Bam*HI-*Hin*dIII subfragment of the 4.3-kb *Xba*I fragment (34); the *Xba*I fragment is also enriched for repetitive sequences contained within an origin-specific DNA fraction (1). Recently, the strand specificity of Okazaki fragment synthesis has been used to map the transition from leading- to lagging-strand synthesis, and thus the origin of bidirectional DNA replication, in the *dhfr* origin region to an 450-bp segment of the 4.3-kb *Xba*I fragment (9a; see Fig. 1).

Strand-specific nucleosome condensation studies in methotrexate-sensitive CHO cells have shown that the origin of replication within the ELF-F/ELF-F′ doublet functions in the absence of gene amplification (23), and transfection experiments with large lambda phage clones have shown that the *dhfr* origin is active when transferred to new chromosomal locations (23). Thus, the *dhfr* origin of replication from the ELF-F/ELF-F′ region represents a suitable model system for delineating initiation of DNA synthesis within a typical mammalian replicon. To identify sequences that may be involved in initiation at the *dhfr* origin, the nucleotide sequence of the origin region was determined, and these sequences then were surveyed for structural elements common to other origins of replication (11). Prominent among the features of the immediate *dhfr* origin region is a fragment of stably bent DNA (11) that includes several consensus sequences for proliferation-specific transcription factors (Fig. 1).

To begin to investigate the mechanism by which the *dhfr* origin of replication is activated, we have used the *dhfr* origin fragment that includes stably bent DNA to assay nuclear extracts from HeLa cells for DNA-binding proteins that display the properties of initiation factors. Here we describe the extensive purification of nuclear protein fractions, which we call RIP60 and RIP100, that contain both origin-specific DNA-binding and ATP-dependent DNA helicase activities.

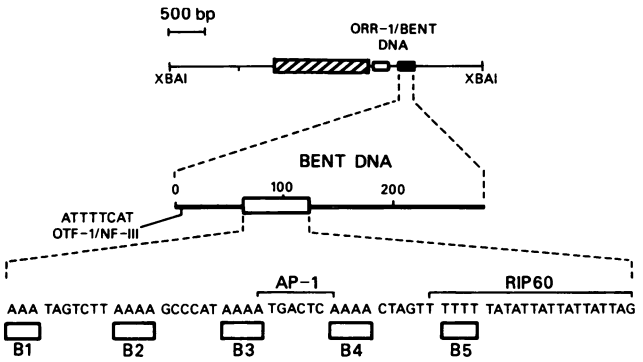

FIG. 1. Demonstration that the *dhfr* origin region contains stably bent DNA. Depicted is a schematic representation of the 4.3-kb *Xba*I *dhfr* origin fragment that encompasses a site for initiation of bidirectional DNA synthesis (▨), the repetitive element ORR-1, and the 280-bp *Hae*III fragment that includes stably bent DNA (for details concerning these DNA sequences, see reference 11). Sequence-directed DNA bending in this region is a result of five oligo(dA)$_{3-4}$ tracts (designated bend elements B1 to B5) phased precisely 10 bp apart. A consensus core binding site for AP1 (TGACTCA) is located between bend elements B3 and B4. The binding site for RIP60 as delineated by DNase I footprinting experiments is indicated (see Fig. 2). Purified OTF1/NFIII recognizes the indicated sequence at the immediate 5′ end of the 280-bp *Hae*III bent DNA fragment.

MATERIALS AND METHODS

DNA plasmids and oligonucleotides. Plasmid pMC304 contains the 280-bp *Hae*III bent DNA fragment of the *dhfr* origin region cloned into the *Sma*I site of pUC12. This fragment spans residues 3342 to 3622 of the *dhfr* origin sequence as reported by Caddle et al. (11); the bend elements B1 to B5 (Fig. 1) occupy residues 3415 to 3459. The bend 208 probe was generated by the polymerase chain reaction and spans residues 3318 to 3526; oligonucleotide ORI spans residues 3432 to 3502; oligonucleotide BEND spans residues 3424 to 3457. Oligonucleotides ORI, BEND, and ATT were synthesized on an Applied Biosystems DNA synthesizer and consist of the following sequences:

ORI (71 bp) 5′-.. ATAAAATGACTCAAAACTAGTTTTTTTATTATTATTATTAGTT...
3′-..GTATTTTACTGAGTTTTGATCAAAAAAATAATAATAATAATCAA...

..CAAATTAGGAAGAAGCTTGCTTTACATG-3′
..GTTTAATCCTTCTTCGAAC.....-5′

BEND (47 bp) 5′..GGGTCTAGAAAAGCCCATAAAATGACTCAAAACTAGTTTTTTCTAGA..-3′
3′..CCCAGATCTTTTCGGGTATTTTACTGAGTTTTGATCAAAAAAGATCT-...5′

ATT (72 bp) 5′-..GGAATTCACTCGGATCCT(AAT)$_{15}$GAGTCGACG..-3′
3′-..... GAGCCTAGGA(TTA)$_{15}$CTCAGCTGC..-5

Oligonucleotide H4 (66 bp) represents sequences from the promoter region of the human histone H4 gene; its sequence is presented as oligonucleotide 1 by Dailey et al. (14). The ARS1-containing plasmid was from John Diffley and Bruce Stillman, Cold Spring Harbor Laboratory; oligonucleotides E1 (28 bp) and E2 (31 bp), used to distinguish ABF1 binding, were from Andrew Buchman, Pennsylvania State University; and oligonucleotide ATT was from Claude Desplan, Rockefeller University.

DNA binding assays. Gel mobility shift assays were performed essentially as described previously (20, 22) and generally used either 0.5 ng of labeled DNA fragment or 0.2 ng of oligonucleotide probe. The amounts of poly(dI-dC) and HeLa nuclear extract or chromatographic fraction included in specific reactions varied; with the 0.35 M KCl DE or 0.5 M KCl S-Sepharose column fractions, 1.0 to 2.0 μl of extract was assayed in the presence of 600 μg of poly(dI-dC) per ml. Assays of fractions from the oligonucleotide columns contained 0.25 to 1.0 μl of protein in the presence of 40 to 150 μg of poly(dI-dC) per ml. For the DNase I footprinting assays, 0.5 ng of the *Eco*RI-*Bam*HI fragment from pMC304, either 3′ or 5′ end labeled at the *Eco*RI site, was used as a probe. Digestion with DNase I was done as described previously (21). The digestion products were analyzed on an 8% polyacrylamide–50% urea gel in 0.5× TBE (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA [pH 8.0]); after autoradiography, the regions protected from DNase I digestion were located by comparison with a G chemical sequencing reaction (35) of the same probe.

Chromatography of HeLa cell nuclear extracts. HeLa nuclear extracts were prepared as described by Digman et al. (18). All chromatography buffers (BC) consisted of 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; 7.9), 20% glycerol, 0.02% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, and different concentrations of KCl. A 100-ml sample of HeLa nuclear extract (12 mg/ml) was loaded onto a 10-ml carboxymethyl (CM)-Sepharose (Pharmacia) column at a flow rate of 12 column volumes per h, and the flowthrough was directly applied to a 40-ml DE-Sepharose (Pharmacia) column at 3 column volumes per h. RIP60 was eluted from the DE-Sepharose column with BC containing 0.35 M KCl, and the pooled step fractions (296 mg of protein) were dialyzed against BC50 (BC plus 50 mM KCl) and applied to a 7-ml S-Sepharose column at 4 column volumes per h. After a wash with 2.5 column volumes of BC50, a 0.2 M KCl step fraction was collected prior to elution of RIP60 with BC500. The pooled 0.5 M KCl fractions (12.2 mg of protein) were dialyzed against BC100 and applied at a rate of 15 column volumes per h to a 1-ml oligonucleotide ATT column. After a wash with 6 column volumes of BC100, 6 ml of BC200 was applied, followed by a 6-column-volume linear salt gradient from 0.2 to 1.0 M KCl. The column was washed with 3 column volumes of 1.0 M KCl to completely elute all of the RIP60 and RIP100 protein. Gradient fractions that exhibited RIP60-binding activity in gel shift assays using oligonucleotide ATT as a probe were pooled, dialyzed against BC100, reapplied to a 0.4-ml oligonucleotide ATT column, and eluted as described above. Protein concentrations were monitored throughout the purification by using the Bio-Rad protein assay. RIP60-binding activity was quantitated by using 0.2 to 2.0 ng of oligonucleotide ATT probe and various dilutions of protein fraction in gel shift assays. Shifted probe was excised from the gel after localization by autoradiography, counted in a Beckman model LS 1801 scintillation counter, and compared (by determining counts per minute) against known amounts of free probe to determine the amount of RIP60-binding activity per unit volume at each step of the purification. Proteins were visualized by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and silver staining, using the Rapid Ag Kit (ICN Biochemicals).

UV cross-linking. UV cross-linking experiments were performed essentially as described by Chodosh et al. (13) and Wu et al. (54), with modifications as described by Dailey et al. (14). The DNA probe was made by annealing the oligonucleotide 5′-CGTCGACTCATTATTATTA-3′ to the top strand of oligonucleotide ATT. The DNA was then filled in with Klenow fragment in the presence of [α-^{32}P]dATP and 50 μM each dCTP, dGTP, and bromo-dUTP. After a 30-min incubation at room temperature, the reaction was chased with 50 μM cold dATP for an additional 30 min. Approximately 0.2 ng of uniformly labeled probe was incubated with RIP60 protein (which had been cycled twice over the oligonucleotide ATT column) and various unlabeled oligonucleotide competitors in a 10-μl reaction volume. After UV irradiation for 20 min and nuclease digestion, the samples were boiled in SDS-PAGE loading buffer and resolved by electrophoresis on a 10% polyacrylamide–SDS stacking gel with molecular weight markers (Sigma 6H). The gel was fixed and stained to visualize the protein markers. The gel was then dried and subjected to autoradiography to visualize labeled polypeptides.

DNA helicase assay. The DNA helicase assay was modified from that described by Lahue and Matson (32). The substrate was prepared by end labeling the universal sequencing primer annealed to single-stranded M13mp18 DNA (Pharmacia) with [α-^{32}P]dATP and Klenow fragment. The labeled DNA hybrid was purified by gel filtration chromatography on Sepharose CL-4B. The standard reaction mixture (20 μl) contained 40 mM Tris hydrochloride (pH 7.5), 2 mM $MgCl_2$, 6 mM dithiothreitol, 50 μg of nuclease free bovine serum albumin (Boehringer Mannheim) per ml, approximately 2 μM helicase substrate, 4 mM ATP, and 1.0 to 1.5 μl of protein extract. Reaction samples were mixed on ice and incubated at 37°C for 15 min, and reactions were then stopped by the addition of 10 μl of 50 mM EDTA–40% glycerol–0.6% SDS–0.1% bromphenol blue–0.1% xylene cyanole. The reaction products were resolved on 8% neutral polyacrylamide gels in 0.5× TBE and visualized by autoradiography.

Covalent labeling with [α-^{32}P]ATP. Covalent labeling of RIP100 was performed essentially as described by Sopta et al. (47). Briefly, 2 μl of protein fraction from the final oligonucleotide affinity column was incubated in a 10-μl reaction volume in the presence of 60 mM KCl, 1 mM $MgCl_2$, 6 mM dithiothreitol, and 1 μl of [α-^{32}P]ATP (specific activity, 800 Ci/mmol; New England Nuclear) for 1 min on ice and 1 min at room temperature before addition of SDS-PAGE loading buffer. For nucleoside triphosphate competition experiments, 0.1 mM unlabeled nucleoside triphosphates were also present in the reaction mixtures. The samples were boiled and resolved by SDS-PAGE along with protein molecular weight markers (Sigma 6H). After electrophoresis, the proteins were fixed and silver stained (ICN Biochemicals) to visualize the molecular weight markers. The gel was then dried and subjected to autoradiography against Kodak XAR-5 film at room temperature.

RESULTS

The *dhfr* origin region contains bent DNA. The *dhfr* origin region was previously surveyed for stably bent DNA by a

two-dimensional gel electrophoresis technique (2). A single 280-bp *Hae*III fragment was identified that migrated approximately 19% slower than predicted in polyacrylamide gels (11). Cloning and DNA sequencing located the bent DNA to the 3′ end of the 1.8-kb *Bam*HI-*Hin*dIII fragment that contains a replication initiation site as identified by in-gel renaturation analysis (34) and strand-specific hybridization studies with Okazaki fragments (9a). Computer-aided examination of these DNA sequences indicated that bending of the 280-bp fragment was likely due to the presence of five periodic tracts of A_{3-4} that are spaced precisely 10 bp apart (sequences labeled B1 to B5 in Fig. 1). To ascertain whether these sequences represented the bending element of the 280-bp *Hae*III fragment, a double-stranded oligonucleotide encompassing sequences B1 to B5 was cloned into plasmid pBEND (57). Cyclic permutation assays (10) confirmed that bend elements B1 to B5 were sufficient to induce stable DNA bending in new sequence contexts. Analysis of the fragment that contains the bent DNA sequences also revealed several nearby consensus sequences for transcriptional activators, including one potential binding site for AP1 (3) and two potential binding sites for OTF1/NFIII. The cellular transcription factor OTF1/NFIII regulates expression of the histone H2B gene during the S phase and also potentiates efficient initiation of adenovirus DNA replication in vitro (39). In gel shift and DNA-binding competition experiments using various portions of the bent DNA fragment as probes, the ATTTTCAT sequence element at the immediate 5′ end of the 280-bp *Hae*III fragment was able to bind purified OTF1/NFIII (Fig. 1), whereas the related sequence, ATTG ACAT, which is located in the reverse orientation near the 3′ end, did not (data not shown). Binding of various purified transcription factors to the consensus AP1 site (ATGACT CA) located between bend elements B3 and B4 will be addressed elsewhere. To identify novel protein factors that interact with other DNA sequences within the 280-bp *Hae*III bent DNA fragment, this fragment was used as a probe in DNase I protection assays using HeLa cell nuclear extracts. A distinct region on both strands was clearly protected from DNase I cleavage (Fig. 2). Interestingly, the protected region, which contained repeated ATT motifs, was located immediately adjacent to the 3′ end of the bent DNA sequences. The position of this binding site was particularly intriguing since it is reminiscent of the structural organization of a variety of well-characterized replication origins in which a specific initiator protein binds adjacent to bent or A+T-rich DNA sequences (19). For this reason, we elected to purify and further characterize the $(ATT)_n$-binding factor.

Purification of the ATT-binding factor, RIP60. The factor that binds the *dhfr* ATT-rich repeats was purified to near homogeneity by a combination of conventional ion-exchange and oligonucleotide affinity chromatography (Table 1). Because competition experiments using the DNase I protection assay showed that an oligonucleotide containing 15 tandem ATT repeats was an effective competitor for binding of the HeLa cell factor, this oligonucleotide (designated ATT) was used to prepare the oligonucleotide affinity column. The ATT-binding factor was purified nearly 9,000-fold, as assessed by quantitative DNA binding assays using oligonucleotide ATT as a probe (Table 1). DNase I footprinting was performed with the most highly purified DNA affinity column fraction on the original *dhfr* bent DNA probe to confirm that the purified factor was responsible for the footprint originally detected in the crude nuclear extract. The pattern of DNase I protection was identical with either nuclear extract (Fig. 2) or the final purified protein preparation (data not shown). After two cycles over the oligonucleotide ATT affinity column, a major polypeptide of approximately 60 kDa that coeluted with the DNA-binding activity was observed on silver-stained SDS-polyacrylamide gels (Fig. 3).

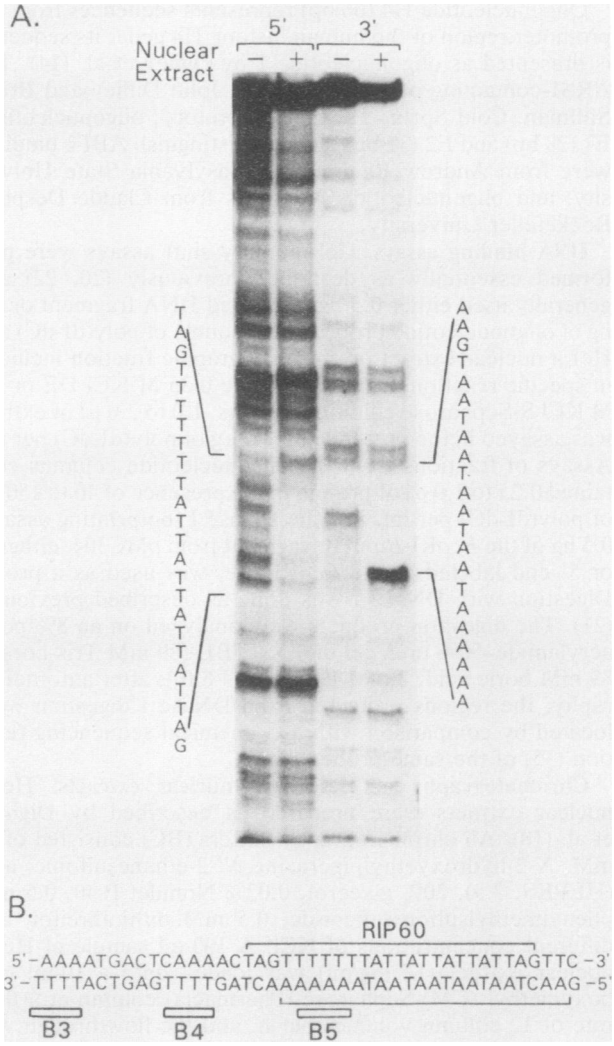

FIG. 2. (A) DNase I footprint analysis of protein-DNA interactions in the bent DNA region. The bent DNA region was excised from plasmid pMC304 and labeled at the *Eco*RI site with either polynucleotide kinase (5′ probe) or Klenow fragment (3′ probe), and the resulting probes were incubated with DNase I in the presence (+) or absence (−) of HeLa cell nuclear extract as described in Materials and Methods. The regions protected from DNase I digestion were located by comparison with a G chemical sequencing reaction of the same probe (not shown) on denaturing polyacrylamide gels. (B) Schematic representation of the DNA sequences protected from DNase I by HeLa cell nuclear extract. The sequences identified by footprint analysis (A) include bend element B5 and an ATT-rich motif that abuts the immediate 3′ end of the bent DNA sequences.

To confirm that the 60-kDa protein represented the ATT-binding activity, UV protein-DNA cross-linking experiments were performed. When proteins bound to uniformly labeled DNA probes are irradiated with UV light, covalent adducts are formed between the amino acids of the bound factor and the radioactive residues of the probe (13, 54). When excess probe that is not directly linked to the bound factor is trimmed away by nuclease digestion, the bound factor

TABLE 1. Purification of RIP60, the ATT-binding activity, from HeLa cell nuclear extracts[a]

Protein fraction	Purification of RIP60			
	Total protein (mg)	Total U	Sp act (U/mg of protein)	Fold purification
Nuclear extract	1,261	42,320	34	1.0
CM flowthrough–0.35 M KCl DE	296	29,274	99	2.9
0.5 M KCl S-Sepharose	12.2	2,271	186	5.5
Oligonucleotide column 1	0.03	1,545	51,500	1,515
Oligonucleotide column 2	0.002	588	294,000	8,647

[a] The DNA-binding activity that recognizes the ATT-rich repeats from the *dhfr* origin region was purified from HeLa cell nuclear extracts by a combination of ion-exchange and DNA affinity chromatography. Purification was monitored by gel electrophoresis mobility shift assays, using oligonucleotide ATT as a probe. One unit of binding activity represents the amount of fraction required to quantitatively bind 10 fmol of probe under gel mobility shift conditions.

remains radiolabeled, and it can be visualized by SDS-PAGE and autoradiography. The specificity of the protein-DNA interaction is assessed by including various DNA competitors during the incubation of the factor with the probe. Specific competitors prevent binding of the factor to the probe and therefore prevent radiolabeling of the protein during UV irradiation.

Hence, an oligonucleotide column fraction that exhibited maximal specific DNA binding was incubated with uniformly radiolabeled oligonucleotide ATT probe in the presence or absence of specific competitor DNA. After UV irradiation and processing of the samples (see Materials and Methods), autoradiography of the SDS-gel revealed several labeled bands in the absence of any competitor (Fig. 4, lane 1). Labeling of a 60-kDa protein was reproducibly prevented by either the oligonucleotide ATT or ORI competitor (Fig. 4, lanes 2 to 5). In contrast, a nonspecific control competitor, oligonucleotide H4, had no effect on labeling of the 60-kDa polypeptide (Fig. 4, lanes 6 and 7). These results establish that the 60-kDa protein that coeluted with the specific DNA-binding activity was indeed the ATT-binding factor. We refer to this 60-kDa origin-binding protein as RIP60.

DNA bending is not sufficient for binding of RIP60. DNA-binding proteins may recognize bent DNA as a structural feature of the DNA rather than as a specific nucleotide sequence per se. To determine whether DNA bending alone was sufficient for RIP60 to bind DNA, we analyzed the ability of various oligonucleotides to compete for RIP60 binding to the *dhfr* origin sequences or oligonucleotide ATT in gel shift assays. Either oligonucleotide ATT (Fig. 5, lanes 2 to 5) or the *dhfr* origin oligonucleotide that includes bend elements B2 to B5 and the ATT repeats (lanes 6 to 9) competed effectively for binding of the purified factor to a 208-bp probe encompassing the bent *dhfr* origin sequences. In contrast, a double-stranded oligonucleotide that spans bend elements B1 to B4 but lacks the ATT repeats (designated BEND) was an ineffective competitor of RIP60 binding even at high concentrations (lanes 10 to 13). Because the ability of oligonucleotide BEND to induce stable DNA bending in new sequence contexts has been independently confirmed by cyclic permutation assays (M. Caddle and N. H. Heintz, unpublished data), we conclude that binding of RIP60 to DNA is directed solely by the tandem ATT repeats and does not require bent DNA. Identical competition profiles were obtained when oligonucleotide ATT rather than the *dhfr* origin sequence was used as a probe (data not shown).

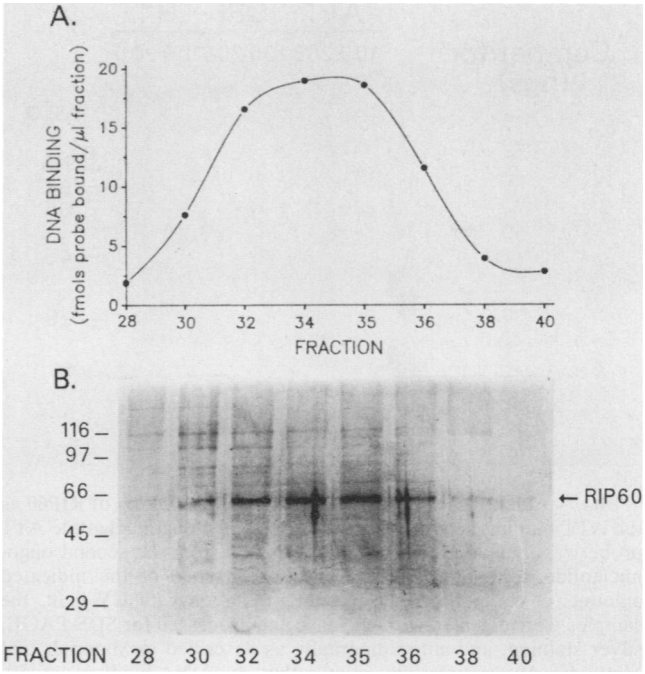

FIG. 3. Elution of ATT-binding activity with the 60-kDa protein, RIP60. The protein factor(s) that binds specifically to the ATT-rich repeats from the *dhfr* origin region was purified from HeLa cell nuclear extracts as shown in Table 1. Column fractions eluted from the second oligonucleotide affinity column by a 0.2 to 1 M KCl gradient that exhibited specific origin DNA binding (A) were analyzed along with protein molecular mass markers (indicated in kilodaltons on the left) by SDS-PAGE and silver staining (B). The 60-kDa polypeptide that coeluted with the ATT-binding activity is designated RIP60.

RIP60 binds to domain B of the yeast origin of replication, ARS1. Because we thought it possible that factors involved in regulating DNA replication may be conserved between species, we sought to determine whether RIP60 would recognize another, well-defined eucaryotic origin of replication. ARS1, an autonomously replicating sequence element of the yeast *Saccharomyces cerevisiae* that has been shown to function as an origin of replication in vivo (reviewed in reference 51), was selected for these experiments. Deletion and point mutagenesis of ARS1 reveal at least three functional domains; domain A contains the 11-bp consensus sequence required for ARS activity (12, 28), domain B contains an A+T-rich region that includes bent DNA and is required for efficient ARS activity under certain growth conditions (45, 53), and domain C contains sequences whose function is incompletely characterized (12).

To determine whether RIP60 binds to ARS1, gel shift and DNase I footprinting experiments were performed with a 311-bp *Hin*dIII-*Eco*RI fragment of ARS1 that includes both domains A and B (Fig. 6A). This probe was incubated with either the S-Sepharose 0.5 M KCl step fraction or oligonucleotide column-purified RIP60 in binding competition experiments. RIP60 exhibited specific binding to ARS1, albeit with an affinity about 10-fold lower than that of either the ATT or ORI DNA probe (Fig. 6B, lanes 2 to 12). Results of the competition assays suggest that the HeLa factor forms multiple specific complexes with the 311-bp ARS1 probe (arrowheads in Fig. 6B).

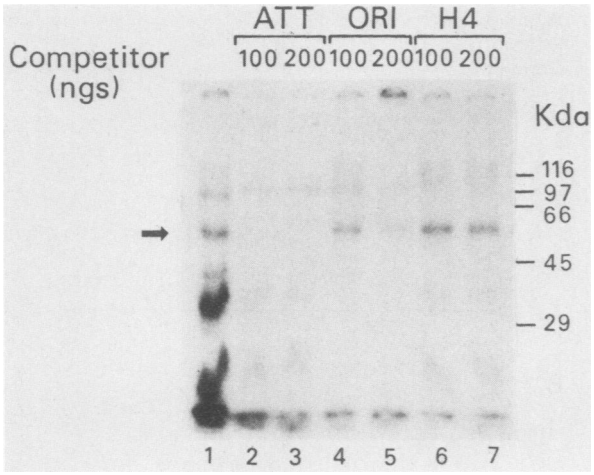

FIG. 4. Identification by protein-DNA cross-linking of RIP60 as the ATT-binding activity. A uniformly labeled oligonucleotide ATT probe was incubated with a RIP60 fraction from the second oligonucleotide column in the presence or absence of the indicated amounts of competitor DNA. After irradiation with UV light, the samples were digested with nuclease and processed for SDS-PAGE, silver staining, and autoradiography as described in Materials and Methods. Autoradiography shows that the DNA-binding activity that was specifically inhibited by either the oligonucleotide ATT (lanes 2 and 3) or ORI (lanes 4 and 5) competitor but not oligonucleotide H4 (lanes 6 and 7) resided in the 60-kDa polypeptide, RIP60 (arrow). Lane 1 shows the cross-linking reaction in the absence of competitor DNA.

To map the position of factor binding to ARS1 more precisely, probes containing either domain A or domain B were generated by digestion with *Bgl*II and *Eco*RI or with *Bgl*II and *Hin*dIII, respectively. RIP60 formed protein-DNA complexes with both the domain A (Fig. 6D) and domain B (Fig. 6C) probes, and these complexes were competed for with the same specificity as those observed for the 311-bp fragment. To better delineate the binding site within domain B, DNase I footprinting experiments were performed under a wide range of conditions. Although binding of RIP60 induced a strong, specific DNase I-hypersensitive site that mapped within the 41-bp bent DNA region of domain B that is known to enhance ARS1 function (arrow in Fig. 6A), we were unable to observe a clear protected region in these experiments (data not shown).

It has been shown that the 3′ end of domain B contains a binding site for ABF1, a yeast factor that binds upstream of several promoters as well as to several ARS elements (reference 8 and references therein). To determine whether RIP60 represents a mammalian homolog of ABF1, binding experiments were performed by using the diagnostic oligonucleotide E1, which contains a consensus wild-type ABF1-binding site, or E2, which contains a consensus binding site for a second yeast ARS-binding factor, GRF1 (8), as a competitor. Neither E1 (Fig. 6E, lanes 5 to 7) nor E2 (lanes 8 to 10) was able to compete for binding of RIP60 to the *dhfr* origin probe, while the domain B fragment of ARS1 competed for binding with an approximately 10-fold-lower affinity than did oligonucleotide ATT (compare lanes 2 and 3 in Fig. 6E).

Despite our inability to observe a discrete footprint in domain B, the gel shift results clearly demonstrate that RIP60 bound specifically to a sequence within domain B of ARS1. Hence, the *Hin*dIII-*Bgl*II domain B restriction fragment was able to compete for RIP60 binding to the ORI oligonucleotide probe, whereas the BEND, H4, E1, and E2 oligonucleotide competitors, even at 20-fold-higher molar concentrations, had no effect on RIP60-domain B interactions. Thus, the competition of RIP60 binding to ARS1 by oligonucleotides ATT and ORI and the competition of RIP60 binding to ORI by the domain B restriction fragment demonstrate that RIP60 binds specifically to ARS1 with about a 10-fold-lower affinity than to either the ATT or ORI probe. These results also show that RIP60 binds to domain B of ARS1 at a site which is distinct from that of ABF1 and that RIP60 is not directly analogous to either ABF1 or GRF1. Although results of the gel shift experiment (Fig. 6D) indicate that RIP60 also recognizes a sequence(s) outside domain B, we have not yet characterized this interaction.

The origin-binding activity of RIP60 cofractionates with a DNA helicase. As described in the introduction, an essential event in the initiation cascade is the localized unwinding of origin DNA by DNA helicases. To determine whether the highly purified fractions containing RIP60 also contained helicase activity, a standard oligonucleotide displacement assay was used (32). The substrate for these experiments

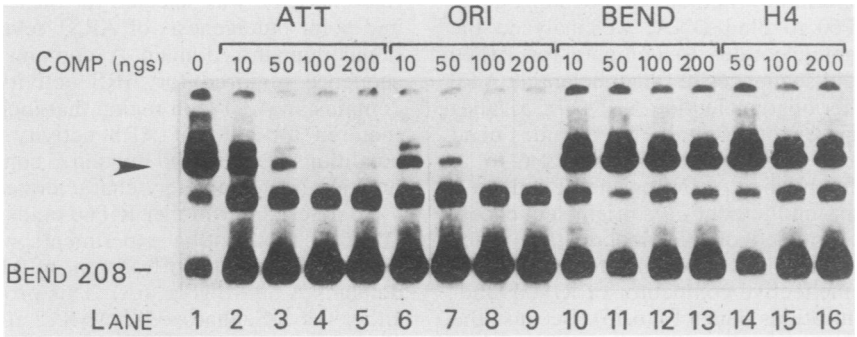

FIG. 5. Demonstration that DNA bending alone is insufficient for RIP60 binding. An end-labeled probe encompassing the *dhfr* bent DNA sequences (bend 208) was incubated with a fraction from the first DNA affinity column that exhibited maximal specific DNA-binding activity, and protein-DNA complexes were resolved by electrophoresis on a 4% neutral polyacrylamide gel. Autoradiography shows that oligonucleotide competitors containing either repeated ATT motifs (lanes 2 to 5) or sequences from the *dhfr* origin region that encompass the RIP60 binding site (lanes 6 to 9) inhibited the formation of specific RIP60 protein-bend 208 DNA complexes (arrowhead). In contrast, neither bent DNA alone (lanes 10 to 13) nor sequences from the human histone H4 promoter region (lanes 14 to 16) could inhibit the formation of RIP60-bend 208 complexes.

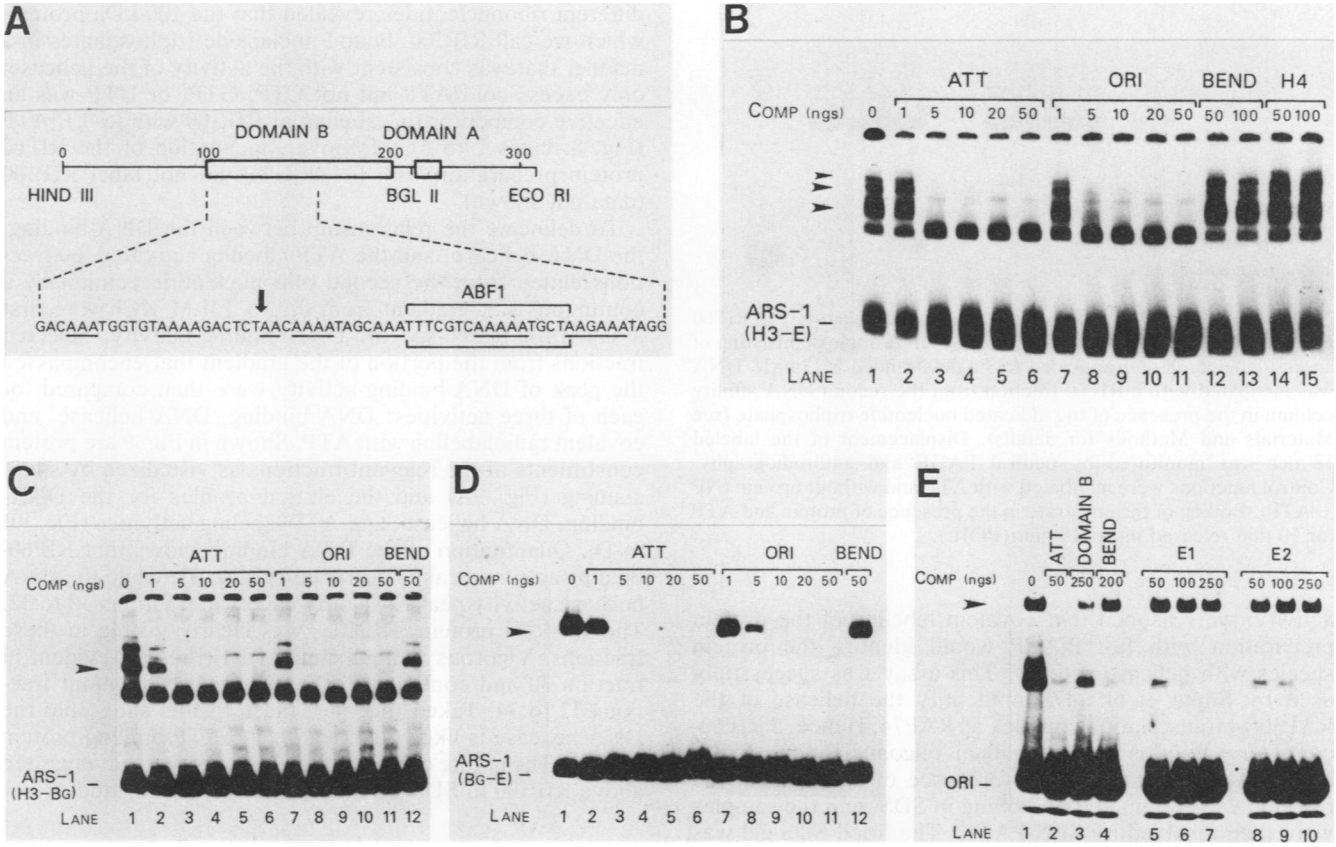

FIG. 6. Binding of RIP60 to domain B of the yeast origin of replication, ARS1. (A) Schematic representation of the 311-bp *Eco*RI-*Hin*dIII fragment of ARS1. Domain A of ARS1 encompasses the 11-bp consensus sequence required for ARS activity (small open box). Domain B (large open box) encompasses a 41-bp region that enhances ARS1 activity under certain growth conditions (45); indicated by solid lines are the oligo(dA) tracts that contribute to sequence-directed DNA bending in domain B. The binding site for the yeast factor ABF1 is also indicated. The vertical arrow indicates the position of a strong DNase I-hypersensitive site observed in footprinting experiments with RIP60 and the *Hin*dIII-*Bgl*II domain B fragment. See text for details. (B to D) Analysis of RIP60-ARS1 interactions by the gel electrophoresis mobility shift assay. Partially purified RIP60 was incubated with the indicated end-labeled probes in the presence of various concentrations of the designated oligonucleotide competitors. Protein-DNA complexes were resolved by neutral PAGE and visualized by autoradiography. Specific RIP60 protein-ARS1 DNA complexes detected in each experiment are indicated by arrowheads. (B) RIP60 binding to the full-length 311-bp *Eco*RI-*Hin*dIII (H3-E) probe of ARS1; (C) RIP60 binding to the *Hin*dIII-*Bgl*II (H3-BG) domain B probe; (D) RIP60 binding to the *Bgl*II-*Eco*RI (BG-E) domain A probe; (E) competition analysis of RIP60 binding. An end-labeled origin (ORI) DNA probe was incubated with partially purified RIP60 and various concentrations of the indicated competitor DNA. Specific RIP60-origin DNA complexes (arrowhead) were visualized by the gel shift assay. Whereas the domain B restriction fragment competed for RIP60 binding with about 10-fold-lower affinity than did oligonucleotide ATT (compare lanes 2 and 3), neither the ABF1-specific oligonucleotide E1 (lanes 5 to 7) nor the GRF1-specific oligonucleotide E2 (lanes 8 to 10) competed for RIP60 binding. DNA bending at ARS1 is addressed in detail by Snyder et al. (45), Williams et al. (53), and Eckdahl and Anderson (19).

was a partially double stranded DNA molecule composed of an end-labeled 18-base oligonucleotide annealed to circular single-stranded M13 DNA. Unwinding of the double-stranded portion of the substrate by a helicase causes release of the oligonucleotide, which can be monitored by electrophoresis of the reaction on nondenaturing polyacrylamide gels and autoradiography. Thus, helicase activity was assessed by incubating the labeled substrate with RIP60-containing fraction from the second oligonucleotide affinity column in the presence or absence of different nucleotides; control reactions were incubated without protein in the presence of ATP.

After incubation at 37°C, displacement of the oligonucleotide was monitored by neutral PAGE and autoradiography as described above. Helicase activity was observed in the presence of RIP60 when ATP or dATP was included in the reaction (Fig. 7). In contrast, no displacement of the labeled oligonucleotide was observed in the absence of protein or in the presence of CTP, GTP, or UTP or their deoxyribonucleotide analogs. These results indicate that both an origin-specific DNA-binding activity and an ATP (or dATP)-dependent DNA helicase activity were present in the most highly purified preparations of RIP60 from the oligonucleotide ATT affinity column.

Covalent radiolabeling of the DNA helicase with ATP. Although the final preparation of RIP60 had been purified more than 8,500-fold, polypeptides in addition to RIP60 were visible on silver-stained SDS-gels (Fig. 3). We therefore sought to determine whether the DNA helicase activity was inherent to RIP60 or could be attributed to another protein. Since the results of the oligonucleotide displacement assays indicated that the helicase requires ATP (or dATP) for

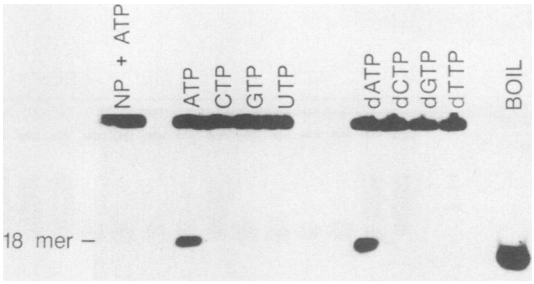

FIG. 7. ATP or dATP-dependent DNA helicase activity in RIP60 preparations. A partially double stranded DNA duplex consisting of an end-labeled 18-mer annealed to single-stranded M13mp18 DNA was incubated with a RIP60 fraction from the second DNA affinity column in the presence of the indicated nucleotide triphosphate (see Materials and Methods for details). Displacement of the labeled 18-mer was monitored by neutral PAGE and autoradiography. Control reactions were incubated with ATP and without protein (NP + ATP). Boiling of the substrate in the presence of protein and ATP for 10 min released intact 18-mer (BOIL).

activity, we reasoned that covalent labeling of the protein preparation with [α-^{32}P]ATP would identify the protein species with helicase activity. This assay was successfully used by Sopta et al. (47) to identify the helicase of the RAP30/74 transcription complex as RAP74. Hence, a RIP60-containing fraction from the final oligonucleotide affinity column was incubated in the presence of [α-^{32}P]ATP, the reaction was terminated by boiling in SDS, and the samples were then resolved by SDS-PAGE. The dried SDS-gel was marked with radioactive ink. The locations of polypeptides within the RIP60 fraction relative to those of protein molecular weight markers was determined by silver staining; labeled proteins were identified by comparing an autoradiograph with the silver-stained gel.

Incubation of the most highly purified RIP60 fraction with [α-^{32}P]ATP resulted in covalent labeling of a single polypeptide of 100 kDa and not of RIP60 (Fig. 8). Competition with

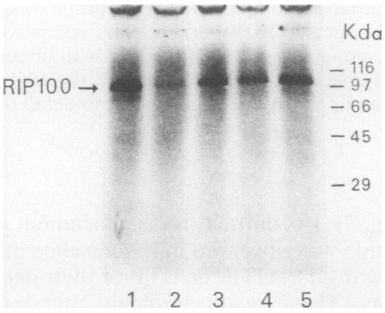

FIG. 8. Identification by covalent radiolabeling with ATP of a 100-kDa polypeptide as the DNA helicase. A RIP60 fraction from the second oligonucleotide affinity column was incubated with [α-^{32}P]ATP in the absence (lane 1) or presence of 0.1 mM unlabeled ATP (lane 2), CTP (lane 3), GTP (lane 4), or UTP (lane 5). After 1 min on ice and 1 min at room temperature, samples were boiled in SDS and resolved by SDS-PAGE. Polypeptides in the RIP60 preparation and protein molecular weight markers were visualized by silver staining (not shown). The SDS-polyacrylamide gel was dried and marked with radioactive ink. Labeled polypeptides were identified by comparing the autoradiograph shown with the silver-stained gel. The 100-kDa polypeptide labeled under these conditions is designated RIP100.

different ribonucleotides revealed that the 100-kDa protein, which we call RIP100, bound nucleoside triphosphates in a manner that was consistent with the activity of the helicase; only excess cold ATP but not CTP, GTP, or UTP was an effective competitor for labeling of RIP100 with [α-^{32}P]ATP (Fig. 8, lanes 2 to 5). Moreover, incubation of the RIP60 protein preparation with [α-^{32}P]GTP did not label RIP100 (data not shown).

To delineate the relationship between the DNA-binding, the DNA helicase, and the ATP-labeling activities, 60 fractions eluted from the second oligonucleotide column by a continuous salt gradient from 0.2 to 1.0 M KCl were first assayed for RIP60-specific DNA-binding activity. Selected fractions from the portion of the gradient that encompassed the peak of DNA-binding activity were then compared for each of three activities: DNA binding, DNA helicase, and covalent radiolabeling with ATP. Shown in Fig. 9 are protein constituents of the relevant fractions as visualized by silver staining (Fig. 9A) and the elution profiles for the DNA-binding, DNA helicase, and ATP-labeling activities (Fig. 9B to D). Quantitation of the DNA binding shows that RIP60-binding activity peaked at fractions 32 to 33, while the DNA helicase activity peaked slightly earlier in fractions 30 to 32. The 100-kDa protein, RIP100, was clearly visible in these fractions. Vigorous radiolabeling of RIP100 was evident in fraction 30 and continued to be observed throughout fractions 32 to 34. Taken together, these results show that the DNA helicase is likely identical to the ATP-binding protein RIP100. The nature of the 116-kDa polypeptide that migrated above RIP100 in SDS-gels has not been investigated.

DISCUSSION

The evidence obtained by extensive origin mapping studies indicates that a chromosomal origin of DNA replication is located within a short region 3′ to the Chinese hamster *dhfr* gene. Although precise definition of the sequences required for *dhfr* origin activity has been thwarted by the lack of suitable genetic or biochemical assays, recent high-resolution mapping experiments using the strand specificity of Okazaki fragment synthesis (9a) have located initiation events at the *dhfr* origin to a region immediately 5′ to the ORR-1 and bent DNA sequences depicted in Fig. 1. This portion of the *dhfr* origin region encompasses several elements characteristic of origins of replication, including stably bent DNA and consensus binding sites for two well-characterized transcription factors associated with cell proliferation, AP1 (3) and OTF1/NFIII (39). These features prompted our search for other nuclear proteins that display the activities expected of factors involved in initiation of DNA replication. In this study, we have characterized a highly purified protein preparation that exhibits both origin-specific DNA-binding and DNA helicase activities; these proteins have been designated RIP60 and RIP100, respectively.

The properties of RIP60 and RIP100 distinguish these factors from two other cellular factors that are known to participate in viral DNA replication in vitro; competition experiments, apparent molecular weight, and chromatographic behavior distinguish RIP60 and RIP100 from both NFI (43) and OTF1/NFIII (reference 39 and references therein). The binding properties of RIP60 also distinguish this factor from alpha-binding protein, a ubiquitous nuclear factor that binds to sequences composed of any combination of six A · T base pairs (46). In addition, competition experiments indicate that RIP60 is not a mammalian homolog of

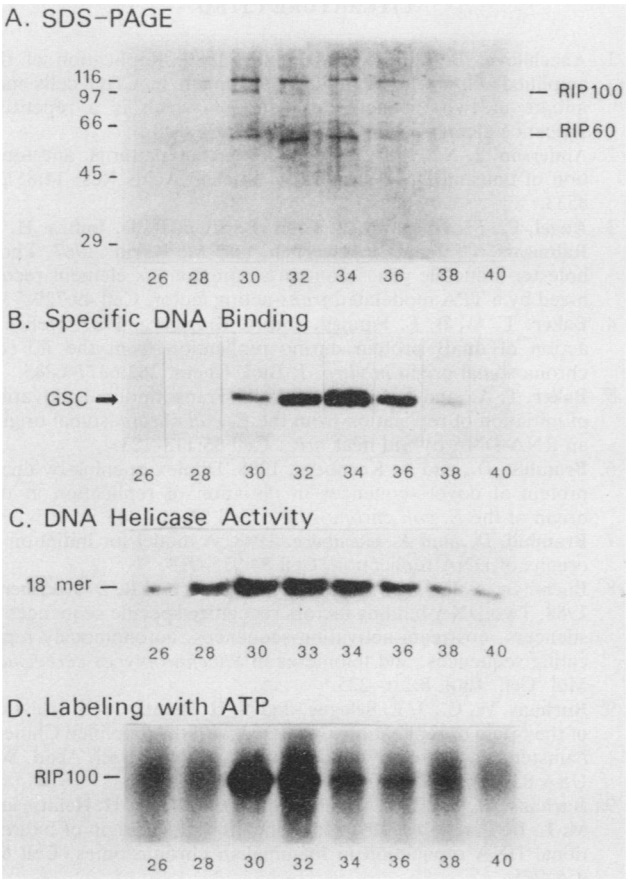

FIG. 9. Purification of RIP60 and RIP100 from HeLa cell nuclear extract. Fractions from the first oligonucleotide affinity column that exhibited maximal RIP60 DNA-binding activity were pooled, dialyzed, and applied to a second ATT DNA affinity column. Fractions eluted by a continuous salt gradient from 0.2 to 1.0 M KCl were first assessed for RIP60 binding by gel shift assays; selected fractions from the portion of the gradient that encompassed RIP60 binding were then analyzed for protein constituents by SDS-PAGE and silver staining (A), specific RIP60 DNA binding by gel mobility shift assays with oligonucleotide ATT as a probe (GSC; B), ATP-dependent DNA helicase activity (C), and covalent labeling with [α-^{32}P]ATP (D). The DNA helicase assay was performed as for Fig. 7; the covalent radiolabeling of RIP100 was accomplished as described for Fig. 8. Molecular mass markers visualized by silver staining are indicated on the left in panel A in kilodaltons.

the yeast protein ABF1 (8). Although the nucleotide requirements for the DNA helicase activity of RIP100 are identical to those of the general transcription initiation factor complex RAP30/74, RIP100 is unlikely to be a portion of RAP30/74 since the transcription factor complex is composed of three polypeptide components of 74, 38, and 30 kDa (47), none of which correspond to the molecular mass of RIP100. Moreover, our covalent radiolabeling experiments with ATP identify a 100-kDa protein as the DNA helicase, whereas the same experiment with RAP30/74 labels a 74-kDa polypeptide (47). Thus, we believe it likely that RIP60 and RIP100 represent novel activities.

Role for RIP60 and RIP100 in DNA replication. While it is not possible at this time to directly assess the role of RIP60 and RIP100 in the initiation of chromosomal DNA replication in mammalian cells, we believe that several of our experimental results strongly support this possibility. First, the RIP60-binding site is located immediately 3′ to the bent DNA sequences within a 20-bp A+T-rich sequence. The position of the RIP60-binding site relative to the bent DNA motif is strikingly reminiscent of the juxtaposition of bent DNA elements and initiation protein-binding sites in a number of well-characterized origins of replication (19). Second, the bent DNA sequences and its attendant RIP60-binding site are located adjacent to the 450-bp region that contains an origin of bidirectional initiation events as defined by strand-specific hybridization studies with Okazaki fragments (9a). Third, RIP60 is able to bind specifically, albeit weakly, to DNA sequences within the functionally important domain B of the yeast origin, ARS1. Fourth, an ATP-dependent helicase activity is recovered with RIP60 after extensive fractionation of HeLa cell nuclear extract.

The purification protocol that we have developed results in a greater than 8,500-fold purification of the *dhfr* origin-specific DNA-binding activity, and UV cross-linking experiments establish that this binding activity resides in the 60-kDa polypeptide, RIP60. Results of the ATP labeling experiments indicate that a 100-kDa polypeptide present in the final protein preparation covalently binds ADP during the hydrolysis of ATP and strongly suggest that the DNA helicase activity that is recovered with the origin-binding activity resides in the 100-kDa protein, RIP100. This interpretation also is supported by competition experiments in which it is observed that ATP, but no other ribonucleotide, is able to inhibit radiolabeling of the 100-kDa protein. Since phosphatases and nucleases in the nuclear extract, CM, DE, and S-Sepharose fractions prohibit quantitation of the RIP100 helicase activity prior to the first oligonucleotide column, we cannot estimate the degree of purification of RIP100 in these experiments. Nevertheless, we believe that the purification of the origin-specific DNA-binding protein, RIP60, with the ATP-dependent helicase, RIP100, after five chromatographic steps is significant and provides evidence that these factors participate in initiation of chromosomal DNA synthesis.

Many important issues concerning RIP60 and RIP100 remain to be resolved. Perhaps most important is the nature of the association of the 60- and 100-kDa polypeptides. Although our data conclusively demonstrate the recovery of both the DNA-binding and DNA helicase activities after five chromatographic steps, we have no definitive evidence that these proteins exist as a specific protein complex in the absence of DNA. If they do not, RIP60 and RIP100 would have had to have been purified separately in the CM, DE, and S-Sepharose chromatography fractions prior to the oligonucleotide ATT column and then formed a complex in the presence of the ATT sequences. We are now attempting to resolve this issue through the use of scanning transmission electron microscopy, velocity sedimentation, and gel filtration analysis, both in the presence and in the absence of specific and nonspecific DNA.

The recovery of an ATP-dependent DNA helicase in our RIP60 and RIP100 preparations is of particular interest. Because a helicase able to unwind eucaryotic origin sequences has not been described, we are now examining the ability of RIP100, in the presence and absence of various purified cofactors, to unwind double-stranded DNA fragments from the *dhfr* origin region. Our preliminary results (P. Held and N. H. Heintz, unpublished data) show that RIP100 helicase activity is stimulated at least 10-fold by single-stranded-DNA-binding proteins and that the RIP100 helicase translocates in 3′ to 5′ direction on single-stranded DNA, as does large T antigen (48).

Initiation of DNA synthesis at the *dhfr* origin of replication. Because it has proven so difficult to assess the activity of mammalian replication origins, one is led to consider that mammalian origins may prove to be multipartite, containing discrete functional and structural elements that may be located at considerable distances from one another. In this regard, we are intrigued by the juxtaposition of a novel repetitive element, ORR-1 (11), consensus binding sites for OTF1/NFIII, AP1, and RIP60, and stably bent DNA within the 450-bp region adjacent to the *dhfr* origin of bidirectional DNA replication. Since it has been shown that binding of RIP60 dramatically enhances DNA bending in the *dhfr* origin region (10), one function of RIP60 may be to foster the interaction of dispersed *cis*-acting elements that regulate origin activity. In this manner, RIP60 may cooperate with other origin-binding factors and the bent DNA sequences to establish a higher-order nucleoprotein complex that is required for origin activation. In this model, the RIP60 and RIP100 polypeptides would then act together to unwind the DNA template during initiation of DNA synthesis.

There is ample precedent for such a mechanism. In *E. coli*, the origin recognition factor DnaA binds to DNA sequences at the chromosomal origin of replication, *oriC*, and potentiates the opening of a local A+T-rich repeated region (7). This protein-DNA complex then serves as a substrate for duplex unwinding by DnaB, the helicase required for initiation of DNA replication (4). A similar series of ordered interactions between an origin recognition protein and the DnaB helicase also mediates initiation of lambda phage DNA replication (44). Interestingly, functional interaction of lambda origin-binding factors is promoted by DNA bending (56). By analogy, it is possible that the DNA-binding activity of RIP60 directs the DNA helicase activity of RIP100 to a higher-order structure at the *dhfr* origin, and both factors then cooperate to unwind the origin during initiation of DNA synthesis.

It is interesting to note the presence of binding sites for OTF1/NFIII and AP1 in the *dhfr* origin region, as an interaction of proliferation-specific transcription factors with initiation proteins during the onset of DNA synthesis would provide a direct pathway for coupling mitogenic signals to the regulation of DNA replication. The observation that members of the family of histone genes that are coordinately regulated during the S phase have distinct subtype-specific transcriptional activators suggests that a pleiotropic signal acts on many constituents that participate in regulating entry into the S phase. Thus, if a role in initiation of DNA synthesis can be established for RIP60 and RIP100, it will be especially interesting to investigate the mechanisms by which these proteins are activated upon entry into the S phase. Identification of a common regulator that activates factors involved in both initiation of DNA synthesis and S-phase-specific gene expression would be a particularly significant advance in our understanding of the mammalian cell cycle.

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